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⑤⑤ Treatment of diseases caused by retroviruses.

⑤⑦ The use of a natural or synthetic oligo- or polysaccharide having at least one S-oxoacid group attached to the saccharic carbon atom through a linking group of lower molecular weight or a pharmaceutically acceptable salt thereof for the manufacture of a medicament for treatment of diseases caused by retroviruses.

**EP 0 240 098 A2**

## TREATMENT OF DISEASES CAUSED BY RETROVIRUSES

### FIELD OF THE INVENTION

The present invention relates to prevention, therapy, etc. of diseases caused by retroviruses. More particularly, the present invention provides a medicament (including veterinary medicament) containing as active ingredient a natural or synthetic oligo- or polysaccharide having at least one S-oxoacid group attached to the saccharic carbon atom through a linking group of lower molecular weight or a pharmaceutically acceptable salt thereof and a method of prevention and therapy etc. of the diseases caused by retroviruses, especially AIDS (acquired immune deficiency syndrome), ARC(AIDS-related complex), PGL (progressive general lymphadenosis) and AIDS-virus carrier using such medicament.

### BACKGROUND OF THE INVENTION

Retroviruses refer to a family of virus which has RNA and reverse transcriptase (RNA-dependent DNA polymerase), which is essential to the first stage of its self-replication for synthesizing complementary DNA on the base of template RNA of the virus.

Retroviruses include various oncoviruses such as avian leukemia virus, avian sarcoma virus, avian reticuloendotheliosis virus, murine mammary cancer virus, murine leukemia virus, murine sarcoma virus, guinea pig type C virus, hamster type C virus, rat leukemia virus, feline leukemia virus, feline sarcoma virus, feline type C virus, ovine leukemia virus, bovine leukemia virus, swine type C virus, simian leukemia virus, Mason-Pfizer virus, simian sarcoma virus, simian T-lymphotropic virus, baboon type C virus, and the like. Among those infective to human, important ones are adult T-cell leukemia virus (ATLV), or human T-lymphotropic virus type I (HTLV-I), and type II (HTLV-II). The adult T-cell leukemia abounds in Japan, especially in the west part, but the effective treatment containing prevention and therapeutics of the disease has not been established.

On the other hand, retroviruses also include those having no oncogenicity, such as visna virus, ovine progressive pneumonia virus, ovine maedi virus, simian T-lymphotropic virus type III (STLV-III), equine infectious anemia virus, and the like. The viruses isolated from human as causative agents for AIDS or ARC etc. (HTLV-III, LAV1, LAV2, ARV, and other so-called AIDS-viruses.) belong to this subfamily. Recently, AIDS-causative viruses are called HIVs (human immune deficiency viruses). Further, as the third subfamily, there is known a spumavirinae to which simian foaming virus belongs. Also, a retrovirus has been isolated recently as a causative virus for Kawasaki disease (mucocutaneous lymphonode syndrome).

World-wide interests have been focused on AIDS due to its unfavorable prognosis. It is a clinical syndrome characterized by recurrent opportunistic infections, (e.g. pneumocystis carinii pneumonia, cryptococcal meningitis, disseminated toxoplasmosis), lymphadenopathy, and an aggressive Kaposi's sarcoma, and induces a high mortality more than 90 % by the dysregulation of immune system. It is also known that the helper-T cells are specifically destroyed by the infection of the virus.

In order to find out pharmaceutical agents effective on the treatment of AIDS, ARC, PGL, and AIDS-virus carrier, the present inventors, using a cell line of MT-4 established from T-cells of adult T-cell leukemia patient and HTLV-III which is a causative virus for AIDS, examined the effects of various substances on the infection and replication of HTLV-III.

The above MT-4 cell line is absolutely susceptible to the infection with HTLV-III followed by causing cell lysis (experimental HTLV-III infection). The present inventors found that when certain polysaccharides having sulfonate group ( $-SO_3^-$ ) or mucopolysaccharides having sulfonate group or their additionally sulfuric acid esterified substances were added to the experimental HTLV-III infection system, the infection of HTLV-III on MT-4 cells and viral replication were strongly inhibited without accompanying any toxicity to the cells.

Further, the present inventors demonstrated that the above polymerized sugar inhibits the reverse transcriptase of the retrovirus in vitro, and thereby suppresses the replication of the virus.

RELATED DISCLOSURES

Among the sulfuric acid esters of polysaccharides, dextran sulfate with lower molecular weight has long been commercialized as an antilipemic or anti-arteriosclerosis agent. Also, dextran sulfate with relatively higher molecular weight is known to have an inhibitory action against herpes virus. (European Patent Laid-Open Publication No.0066379). However, since the herpes virus is a DNA virus, its replication is absolutely different from that of the retrovirus which depends entirely on reverse transcriptase for synthesis of DNA. Accordingly, the effectiveness on herpes virus does not necessarily mean the effectiveness on retrovirus alike. Furthermore, dextran sulfate with lower molecular weight less than 10,000 was found to be almost ineffective on herpes viruses.

Among the mucopolysaccharides or these sulfates, chondroitin sulfate is commercialized as a drug for sensorineural hearing impairment, neuralgia, lumbago and chronic nephritis, and also as a cornea-protective ophthalmic solution. Keratan sulfate is obtainable from the cartilage, teichronic acid from the cell walls of *Bacillus subtilis*, hyaluronic acid from shark skin, whale cartilage, or from human serum, heparan sulfate from bovine liver or lung, and chitin from arthropod or from fungus or yeast, respectively. The preparation process for the further sulfuric acid esterified compound of chondroitin sulfate is described in Japanese Patent Publication (JP, B2) No.9570/1971.

Heparin is known to inhibit various enzymes *in vitro*, e.g., DNA polymelase of phytohemagglutinin stimulated human lymphocytes and reverse transcriptase of simian sarcoma virus (Cancer research, 38, 2401 - 2407), but is not proved to inhibit the viral infection of cells.

SUMMARY OF THE INVENTION

In one aspect, the present invention provides a method of treatment of diseases caused by retroviruses which comprises administering an effective amount of a natural or synthetic oligo- or polysaccharide having at least one S-oxoacid group attached to the saccharic carbon atom through a linking group of lower molecular weight or a pharmaceutically acceptable salt thereof to a subject in need of such treatment.

In another aspect, the present invention provides a use of the above oligo- or polysaccharide or a salt thereof for the manufacture of a medicament for treatment of diseases caused by retroviruses.

In a further aspect, the present invention provides a pharmaceutical composition comprising the above oligo- or polysaccharide or a salt thereof as an active ingredient in association with a pharmaceutically acceptable carrier, diluent or excipient.

BRIEF EXPLANATION OF THE DRAWINGS:

Figs. 1 - 7 show the reverse transcriptase inhibition activities of the test substances in Example 1.

Fig. 8 shows the reverse transcriptase inhibition activity of the test substance in Example 2.

Figs. 9 - 15, 16 - 22, and 23 - 29 show the effects of the test substances on cell growth, viability, and infected cell rate (%) of MT-4 cells infected with HTLV-III, respectively, in Example 3.

Figs. 30 - 33 show the reverse trascriptase inhibition activities of the test substance in Example 5.

Figs. 34 - 37, 38-41, 42 - 45 show the effects of the test substaces or cell growth, viability, and infected cell rate (%) of MT-4 cells infected with HTLV-III, respectively, in Example 6.

Figs. 46 - 48 show the effects of heparin on cell growth, viability and infected cell rate of MT-4 cells infected with HTLV-III, respectively, in Example 7.

DETAILED DESCRIPTION AND PREFERRED EMBODIMENT

The term "treatment" herein is intended to cover all controls of disease including prevention, sustantion (i.e. prevention of aggravation), reducing (i.e. alleviation of conditions) and therapy.

The retroviruses includes all viruses having RNA and reverse transcriptase as the basic components including those exemplified above.

The diseases referred to herein cover all the diseases caused by retroviruses, including those bearing or not bearing the aforementioned virus name. Important diseases are the diseases caused by AIDS-viruses.

The oligo-or polysaccharide usable in the present invention are those having at least one S-oxoacid group attached to the saccharic carbon atom through a linking group of lower molecular weight. Such oligo-or polysaccharide may be natural or synthetic. The term "natural" is intended to mean that the oligo-or polysaccharide is obtainable from a natural source such as plant, microorganism or animal by extraction and other means. The term "synthetic" is intended to mean that the oligo-or polysaccharide is obtainable synthetically, for example, by introducing S-oxoacid group into other oligo-or polysaccharide which has or has not S-oxoacid group and which is natural or unnatural (and synthetic).

The term "oligosaccharide" refers to a carbohydrate containing from two up to about nine monosaccharides linked together. For example, when a oligosaccharide contains three monosaccharides, one, two or three of the monosaccharides may have at least one S-oxoacid group.

The term "polysaccharide" refers to a carbohydrate containing about ten or more monosaccharides linked together. At least one and a minor or major part or the all of the monosaccharides may have at least one and normally up to four S-oxoacid groups.

The S-oxoacid group includes sulfo group ( $-\text{SO}_3\text{H}$ ) and hydroxysulfinyl group ( $-\text{SO.OH}$ ). Preferable S-oxoacid group is sulfo group.

The term "saccharic carbon atom" refers to a carbon atom which is a member of tetrahydrofuran or tetrahydropyran ring of monosaccharide contained in the oligo-or polysaccharide.

The linking group of lower molecular weight includes oxy ( $-\text{O}-$ ), imino ( $-\text{NH}-$ ), thio ( $-\text{S}-$ ), methylene ( $-\text{CH}_2-$ ), ethylidene ( $-\text{CH}(\text{CH}_3)-$ ) groups and the like. The term "lower molecular weight" is intended to mean that the group has a molecular weight from about 14 up to about 32. Preferable linking group is oxy and imino groups.

One class of the oligo-or polysaccharide is a natural polysaccharide having at least one hydrogen sulfate group ( $-\text{O}-\text{SO}_3\text{H}$ ) and is obtained from a plant or a microorganism, or a synthetic polysaccharide having at least one hydrogen sulfate group ( $-\text{O}-\text{SO}_3\text{H}$ ) and is formed by esterifying a polysaccharide obtained from a plant or a microorganism.

Within this class, a preferable subclass is polysaccharide composed of non-amino monosaccharide (including sugar acid) as the repeating unit. This polysaccharide, however, may contain a trace amount of nitrogen. Examples of the non-amino sugar repeating units include xylose, arabinose, rhamnose, fucose, glucose, galactose, glucuronic acid, galacturonic acid, mannuronic acid, etc. The natural polysaccharide includes carrageenan (galactan sulfate obtainable from *Gigartina tenella*, etc.) and fucoidin (polyfucose sulfate obtainable from *Laminaria* brown seaweed). Carrageenan includes  $\alpha$ -carrageenin,  $\lambda$ -carrageenan,  $\iota$ -carrageenan, etc. which have the different content of hydrogen sulfate group. The synthetic polysaccharide includes those to be obtained by sulfuric acid esterification of polysaccharides, e.g., starch and partial hydrolyzate thereof, dextran which is produced by *Leuconostoc* sp. and partial hydrolyzate thereof (usually having the molecular weight of 500 - 2,000,000, ordinarily 2,000 and 300,000, preferably 2,000 - 10,000, most suitably 3,000 - 8,000, e.g., 7,000 - 8000), glycogen, pectin, cellulose, plant viscous liquids (gum arabic, tragacanth gum, etc.), plant mucilage products (those obtainable from *Hibiscus esculentus*, *Aloe*, *Brasenia schreberi*, etc.), viscous liquids of marine and fresh water algae (alginic acid, laminarin, etc.) or polysaccharide derived from microorganism (lentinan, pluron, mannan, etc.). They include known ones (dextran sulfate, cf., European Patent Laid-open Publication No.0066379) and novel ones. The novel ones may be produced in the same manner as in the known ones. An example of the preparation process is shown, as follows:-

Chlorosulfonic acid is added dropwise to dry pyridin of 8 - 10 fold volume while cooling. To the mixture are added small amounts of formamide and dextran (about 1/4 weight of chlorosulfonic acid), and the mixture is heated to 55 - 65° C under stirring. After stirring the mixture for several hours, the solvent is distilled off, and the residue is purified for example by recrystallization, dialysis, etc. Within the synthetic polysaccharide, those obtained by further sulfuric acid esterification is represented by the term "polysulfate".

Another class of the oligo-or polysaccharide is a natural polysaccharide having at least one hydrogen sulfate group ( $-\text{O}-\text{SO}_3\text{H}$ ) and is obtained from an animal, or a synthetic polysaccharide having at least one hydrogen sulfate group ( $-\text{O}-\text{SO}_3\text{H}$ ) and is formed by esterifying a polysaccharide obtained from an animal.

Within this class, a preferable subclass is mucopolysaccharides, which is composed of amino monosaccharide (including N-acyl or  $\text{NH}-\text{SO}_3\text{H}$ ) as the repeating unit. This may further contain as another repeating unit non-amino sugar or an acid derivative thereof. The repeating amino-sugar unit or its N-acylated (preferably N-acetylated) derivatives include glucosamine, galactosamine, N-acetylated derivatives of them, and sulfuric acid ester or partial hydrolyzate of the above compound. Examples of monosaccharide or acid (preferably, hexulonic acid) includes glucose, galactose, glucuronic acid, iduronic acid, etc. The

mucopolysaccharides containing such repeating unit include heparin, keratan sulfate, chondroitin-4-sulfate, chondroitin-6-sulfate, dermatan sulfate, teichuronic acid, hyaluronic acid, heparitin sulfate, chitin, and their partial hydrolyzates, modified derivatives (e.g., partial acylated products), and synthetic polysaccharides containing the repeating unit such as above.

5 The mucopolysaccharide polysulfates are defined as the products which are synthesized by additional sulfuric acid esterification of the above mucopolysaccharides having sulfate group. This esterification may be carried out, for example, according to the procedure described in Japanese Patent Publication No.9570/1971. In general, the esterification is carried out by treatment of the mucopolysaccharides with one of sulfating reagents such as concentrated sulfuric acid or chlorosulfonic acid.

10 These reactions are usually carried out with or without a solvent at low temperature. The reaction product is separated by conventional procedure, e.g., neutralization, concentration, precipitation, recrystallization, chromatography, etc.

The term "pharmaceutically acceptable salt" is intended to mean that the salt has the biological activity of the parent compound and is not unusably toxic at the administration level. Such salt includes the salt of inorganic base such as sodium salt, potassium salt, ammonium salt, etc., and salt of organic base such as diethanolamine salt, cyclohexylamine salt, amino acid salt, etc. These salts are produced from the corresponding acids by the conventional procedures. The above oligo- or polysaccharides and their salts may be solely used or as a mixture with the metal salts such as zinc, aluminum, etc. The oligo- or polysaccharide should be administered at a dose sufficient to produce the effect for the desired treatment. For example, the dosage of the sulfates of the above polysaccharide or their salts sufficient to produce blood concentration for anti-virus activity is generally 0.2 - 200 mg/kg, preferably 0.5 - 100 mg/kg. In the case of human, an amount of about 10 mg - 10 g/day, preferably about 50 mg - 5 g/day, is administered in 1 - 4 divisions a day, or as a sustained release form. The administration route can be optional such as oral, rectal, nasal, local (including sublingual), injection (including subcutaneous, intracutaneous, intramuscular and intravenous), inunction etc. Preferable route is selected depending on various factors including kind of active ingredient, conditions and age of patient, severity of infection etc.

25 The dosage of the mucopolysaccharides or their polysulfate or the salts thereof sufficient to produce a concentration for anti-virus activity is generally 0.2 - 200 mg/kg, preferably 0.5 - 100 mg/kg. In the case of human, an amount of about 10 mg - 20 g/day, preferably about 50 mg - 10 g/day is administered in 1 - 4 divisions a day, or as sustained release form.

30 The administration route can be optional such as oral, local, injection, inunction, etc.

For administration, the effective ingredient may be mixed with a pharmaceutical carrier such as organic or inorganic solid or liquid excipient e.g. suitable for internal administration or injection, and administered in the form of the conventional pharmaceutical preparation. Such preparation includes solids (e.g., tablet, granule, powder, capsule, etc.) and liquids (e.g., liquid, emulsion, suspension, etc.), and ointment. The above carriers include starch, lactose, glucose, sucrose, dextrin, cellulose, paraffin, fatty acid glyceride, water, alcohol, gum arabic, etc. If necessary, auxiliary, stabilizer, emulsifier, lubricant, binder, pH regulating agent, isotonicity agent, and other additives in ordinary use may be added.

40 The toxicity of the above oligo- or polysaccharide is extremely low. For example, the acute toxicity ( $LD_{50}$ ) of sodium dextran sulfate (molecular weight 7,000 - 8,000, S-content 17 - 20 %) is 21,000 mg/kg when orally, and 4,500 mg/kg when intravenously administered to mice. The acute toxicity ( $LD_{50}$ ) of sodium chondroitin sulfate is 4,000 mg/kg or more when intraperitoneally, and 7,500 mg/kg or more when orally administered to mice. The acute toxicity ( $LD_{50}$ ) of sodium heparin is 1,500 - 2,000 mg/kg when intravenously injected to mice.

### 45 Examples

The following examples will illustrate the present invention in further detail.

### 50 Preparation 1

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## Preparation of chondroitin polysulfate from chondroitin sulfate

Chondroitin sulfate (5g) was added to 95 % sulfuric acid (10 ml) cooled at below -25°C with stirring. After addition, the reaction mixture was stirred at the same temperature for 90 minutes. After the end of the period, the reaction solution was gradually poured onto ice (120 g) with stirring. To the resulting solution was gradually added calcium carbonate with well stirring. The precipitates were filtered off, which, then were washed well with water. To the combined filtrates (240 ml) was added ethanol (60 ml), and the solution was kept to stand overnight at 5 °C to precipitate calcium sulfate. The precipitates were filtered off, and the filtrate was adjusted to pH 10 with sodium carbonate. After addition of acetic acid to make the solution weakly acidic, the solution was concentrated to about 20 ml, then diluted with ethanol (100 ml), and kept to stand overnight at 5 °C. The precipitates in the solution were isolated with centrifugation, washed with ethanol, and with ether, and dried under vacuum to give the white powder of the title compound.

15 Preparation 2

Preparation of keratan polysulfate from keratan sulfate.

Preparation 1 was repeated except that keratan sulfate (100 mg) is used as a starting material and 1 ml in place of 10 ml of 95% sulfuric acid is used, to give the title compound.

Formulation 1 Sodium dextran sulfate (molecular weight: 7,000 - 8,000, S-content: 17 - 20%) 150 mg.  
 Corn starch 45 mg  
 25 Lactose 300 mg  
 Magnesium stearate 5 mg

The above ingredients are mixed, granulated, and pressed according to the conventional procedure to make tablets, which were then enterically coated.

Formulation 2 Sodium dextran sulfate (molecular weight: 7,000 - 8,000, S-content: 17 - 20%) 600 mg  
 Physiological saline q.s. to 10 ml.

Formulation 3 Sodium dextran sulfate (molecular weight: 5,000, S-content: 13 - 14%) 600 mg  
 Physiological saline q.s. to 10 ml.

Formulation 4 Sodium salt of chondroitin sulfate 150 mg  
 Corn starch 45 mg  
 Lactose 300 mg  
 45 Magnesium stearate 5 mg

The above ingredients are mixed, granulated, and pressed according to conventional procedure to make tablets, which were then enterically coated.

Formulation 5 Sodium salt of keratan polysulfate 400 mg  
 Lactose 195 mg  
 Magnesium stearate 5 mg

The above ingredients are mixed according to the conventional procedure and filled in hard gelatine capsules.

5 Formulation 6 Sodium salt of chondroitin polysulfate 300 mg  
Physiological saline q.s. to 10 ml.

10 Formulation 7 Sodium heparin 25,000 units  
Physiological saline q.s. to 10 ml.

Formulation 8 Calcium heparin 5000 units  
Procain hydrochloride 10 mg  
15 Water q.s. to 10 ml

#### Example 1 (Inhibition of reverse transcriptase activity)

20 Test substances were assayed for inhibition against the enzyme activity of reverse transcriptase (authentic sample) derived from Avian Myeloblastosis Virus (abbrev. AMV), a kind of retrovirus. Five microliters of  $(\gamma A)_n$  (template RNA), 4  $\mu$ l of  $(dT)_{12-18}$  (primer DNA), and 1  $\mu$ l of water were mixed with 5  $\mu$ l of 0.5M Tris-HCl (pH 8.4) including 0.1 % triton X-100, 5  $\mu$ l of 1mM-MgCl<sub>2</sub>, 5  $\mu$ l of 20 mM-DDT, 5  $\mu$ l of water, and [<sup>3</sup>H]-TTP (tritium labeled thymidine triphosphate). To this mixture, test substances in solutions  
25 (final concentrations: 1, 0.1, and 0.01  $\mu$ g/ml, 5  $\mu$ l) or buffer solutions (control, 5  $\mu$ l) at various doses were added. Then, 5  $\mu$ l (one unit) of the authentic reverse transcriptase derived from AMV was added and the reaction mixture was incubated at 37°C for 30 minutes. The reaction was stopped by addition of trichloroacetic acid, and after filtering the reaction mixture, the radioactivity of the polymerized (<sup>3</sup>H-T)<sub>n</sub> retained on the filter was measured using a liquid scintillation counter. As the test substances, sodium  
30 dextran sulfate (molecular weight: 5000), same (molecular weight: 8000), same (molecular weight: 500000), fucoidin, x-carrageenan,  $\lambda$ -carrageenan, and i-carrageenan were used. The results are shown in Figs. 1-7.

Figs. 1-7 show that the enzyme inhibition increases with the increasing doses of the above test substances.

#### 35 Example 2 (Inhibition of reverse transcriptase activity)

The assay procedure of Example 1 was repeated using disrupted HTLV-III virions as a crude reverse transcriptase in order to evaluate the reverse transcriptase inhibitory effect of dextran sulfate (DS, molecular  
40 weight 7,000 - 8000, S-content 17 - 20 %). The result is shown in Fig. 8.

Fig. 8 shows that DS has an inhibitory effect against the reverse transcriptase derived from AIDS-virus, HTLV-III.

#### 45 Example 3 (Anti-AIDS virus activity)

To MT-4 cells (30 × 10<sup>4</sup>/ml) cultured in RPMI-1640 medium containing 10 % bovine serum, was inoculated HTLV-III, and the suspension was incubated at 37 °C for 1 hour to cause the adsorption of the virus. The cell : virus ratio was 500:1. The cells were then washed, and cultured with or without various  
50 doses of the test substances (same as those of Example 1) at 37 °C under 5 % CO<sub>2</sub> for 3 days, after which cell growth, viability, and percentage of infected cells were recorded. The infected cells were distinguished from the uninfected cells by indirect immuno-fluorescence method. Thus ; the cultured cells were fixed with cold methanol on a slide glass, reacted with antibody to the HTLV-III-specific antigens, and further with the secondary antibody (having fluorescent label). The results are shown in Figs. 9-29, wherein,  $\nabla$ ,  $\Delta$  and  
55 show the controls without virus,  $\nabla$ ,  $\Delta$  and show the infection experiments with HTLV-III. The cell growth is indicated in number of cells, the viability (%) in number of viable cells × 100/number of total cells, and the infected cell rate (%) in number of fluorescent-positive cells × 100/number of total cells.

Figs. 9-22 demonstrate that when no test compound was added to the medium, the cells did not grow and were killed by viral infection, whereas depending on the increase in the dose of the test substance, the number of cells and viability came near to the values of the control without virus. Also, it is shown from Figs. 23 - 29 that when the test substance is not added, almost all cells are infected (~100%), whereas depending on the increase in the dose of the test substances, the infection of cells was strongly inhibited.

Accordingly, it is evident that the test substances have excellent inhibiting activities against infection of AIDS virus to host cells and viral proliferation.

#### 10 Example 4 (Cytotoxicity)

As the anti-virus substances often show toxicity to the host cells, the following experiment was conducted to know whether or not the test substances (used in Example 1 and 3) would induce cytotoxicity.

15 MT-4 cells were cultured with or without 1 - 100  $\mu\text{g/ml}$  of each test substance which is the same as in Example 1 and 3 and the proliferation and viability of cells were recorded. The results are shown in the following Table.

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Substance		
( $\mu\text{g/ml}$ )	Cell number	Viability
	( $\times 10^4$ cells/ml)	(%)
Sodium dextran sulfate (molecular weight: 5000, S-content: 13%)		
100	121	93
10	127	92



5	1	123	90
	0	124	87
	Sodium dextran sulfate (molecular weight: 7000 - 8000,		
10		S-content: 17 - 20%)	
	100	130	94
	10	138	91
15	10	120	90
	0	124	87
20	Sodium dextran sulfate (molecular weight: 500,000		
		S-content: 16%)	
	100	126	86
25	10	139	94
	1	124	88
30	0	124	87
	Fucoidin		
	100	71	93
35	10	112	99
	1	141	92
40	0	124	87
	κ-Carrageenan 80 % + λ-Carragheenin 20 %		
	100	111	92
45	10	149	93
	1	147	93
50	0	124	87
	λ -Carrageenan		
55	100	83	94

	10	203	94
	1	147	89
5	0	124	87
	1-Carrageenan		
10	100	144	80
	10	128	93
	1	135	94
15	0	124	87

20 The above results show that the test substances have little cytotoxicity.

#### Example 5 (Inhibition of reverse transcriptase activity)

25 The effects of the test substances on the reverse transcriptase activity of AMV were evaluated by the method described in Example 1. The test substances used are chondroitin sulfate (S-content: 6.2 - 6.9 %), chondroitin polysulfate (S-content: 11.6 - 12.1 %), keratan sulfate (S-content: 7.0 - 8.0 %), and keratan polysulfate (S-content: 9.7 %). The results are shown in Figs. 30 - 33.

30 Figures 30 - 33 indicate that the enzyme inhibition increases with the increasing doses of the above test substances. The above results also demonstrate that the reverse transcriptase inhibitory activity of the test substances is closely related to the number of sulfate group in the molecule, as evidenced by the fact that the synthetic substances (e.g. chondroitin polysulfate and keratan polysulfate) have stronger activity than the natural substances (e.g. chondroitin sulfate and keratan polysulfate).

#### Example 6 (Anti-AIDS virus activity)

35 Test substances were assayed for the anti-AIDS virus activity in the same manner as in Example 3 using cell culture. The test substances are the same as those used in Example 5. The results are shown in Figs. 34 - 45, wherein ▽, Δ and □ show the controls without virus, ▼, ▲ and ■ show the infection tests with virus.

40 Figs. 34 - 41 demonstrate that, without the test substances, the cells did not grow and were killed by viral infection, whereas depending on the increase in the dose of the test substance, the decrease in number of cells and loss of viability were prevented. Also, Figs. 42 - 45 demonstrate that when the test substance was not present, almost all of the cells were infected (≈ 100%) with HTLV-III, whereas depending on the increase in the dose of the test substances, the infected cell rate (%) was significantly reduced.

45 The above results also indicate that the synthetic mucopolysaccharide polysulfates having higher S-content had stronger anti-AIDS virus activities than those of the natural products.

#### Example 7 (Anti-AIDS virus activity)

50 The anti-AIDS virus activity of heparin was evaluated in the same manner as in Example 3. The results are shown in Figs. 46 - 48, wherein ▽, Δ and □ show the controls without virus, and ▼, ▲, and ■ show the infection tests with virus.

Figs. 46 and 47 show that without heparin, the cells did not grow and were killed by viral infection, whereas depending on the increase in the dose of heparin, the number of cells and viability were maintained to that of control. It was also shown from Fig. 48 that when heparin was not present, almost all of the cells are infected, whereas depending on the increase in the dose of heparin, they become less susceptible to the viral infection.

#### Example 8 (Cytotoxicity)

As the anti-virus substances often show toxicity to the host cells, experiment was conducted to know whether or not heparin would induce such cytotoxicity.

Without the virus, MT-4 cells were cultured in the same manner as in Example 4 except the test sample was heparin, and proliferation and viability of cells were recorded. The results are shown in the following Table.

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20	Heparin ( $\mu\text{g/ml}$ )	Cell number ( $\times 10^4$ cells/ml)	Viability (%)
25	100	133	94
	10	142	89
	1	143	91
30	0	124	87

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The above results show that heparin has little cytotoxicity.

#### Example 9 (Anti-AIDS activity)

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In order to examine the correlation between the anti-AIDS virus activity and the molecular structure (especially, molecular weight and S-content or number of sulfate group in this case) of various test substances including those used in Examples 1 - 7, the anti-AIDS activities were evaluated for the various naturally occurring polysaccharides, polysaccharides having sulfate group, mucopolysaccharides, mucopolysaccharide sulfate, and mucopolysaccharide polysulfate. Further, similar experiments were carried out with various other sulfates which were synthetically obtained. The experimental procedures employed are identical to that in preceding experiments. The cultured MT-4 cells were infected with HTLV-III and the inhibitory effects of various test substances on the infected cell rate (number of fluorescent cell  $\times$  100/total cell, %) were determined at 6th day. The results are shown in the following Table.

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1) Dextran, their synthetic sulfates, and monosaccharides having sulfate groups.

Test substance	Molecular weight	S-content (%)	Infected cell (%) *		
			10 µg/ml	100 µg/ml	1000 µg/ml
Dextran	9,000	0	100	100	100
"	300,000	0	100	100	100
Dextran sulfate	5,000	≈ 13	18	0	0
"	8,000	≈ 14	25	0	0
"	500,000	≈ 16	20	0	0
"	7,000-8,000	17-20	1	0	0
"	3,500	6	100	82	1
(Monosaccharides)					
Glucose-6-sulfate		12	100	100	100
Glucose-polysulfate		22	100	100	100
N-acetylglucosamine polysulfate		18	100	100	100

\* The control without test substance shows the value of 100% as the infected cell rate under the same conditions.

## 2) Polysaccharides derived from algae and their sulfates.

Test substance	Molecular S-content		Infected cell (%)	
	weight	(%)	10µg/ml	100µg/ml
κ-Carraheenan		≈ 7	95	1
γ- "		≈ 16	3	1
ι- "		≈ 12	100	31
Fucoidan		≈ 15	32	1
Agarose	60,000 - 180,000	2 - 3	100	100
Alginic acid	32,000 - 240,000	0	100	100
Alginic acid sulfate	50,000 - 300,000	14	7	4

## 3) Chitin and chitosan and their sulfates

Test substance	Molecular weight		S-content (%)		Infected cell (%)	
Chitin			0	100	100	100
Chitin sulfate			9	100	81	
Chitosan			0	100	100	
Chitosan sulfate			18	1	1	

## 4) Mucopolysaccharides derived from animals, and their sulfates and polysulfates

Test substance	Molecular weight		S-content (%)		Infected cell (%)	
Chondroitin	25,000 - 30,000		- 0	100	100	
Chondroitin polysulfate	5,000 - 8,000		13	3	1	
Chondroitin-4-sulfate	30,000 - 50,000		6	90	80	
Chondroitin-4-sulfate polysulfate			16	2	1	
Dermatan sulfate	20,000 - 40,000		6	100	80	
Chondroitin-6-sulfate	30,000 - 50,000		6	100	100	

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Chondroitin-6-sulfate polysulfate	15	2	1
Heparin	13	41	1
	7,000 - 30,000		
Heparitin sulfate	7	100	90
	15,000		
Keratan sulfate	7	100	60
	4,000 - 20,000		
Keratan polysulfate	10	40	20
Hyaluronic acid	0	100	100
	10,000 - 100,000		
Hyaluronic acid sulfate	8	100	70

## 5) Other polysaccharides

None of pectin, coromic acid, inulin, raffinose, and methylcellulose showed any anti-AIDS virus activities.

From the above results, it can be clearly seen that the anti-AIDS virus activity is closely related to the S-content or number of sulfate group in this case rather than to the molecular weight. Substances without sulfate group showed no anti-AIDS activity. Further, the anti-AIDS activity was intensified with increasing S-content (number of sulfate group) of the molecule. With respect to the relation with the molecular weight, there was no effect at all in the monosaccharides. However, in the substances having molecular weights of 5,000 and higher, the increase of the molecular weight did not affect the anti-AIDS virus activity as seen in e.g. dextran sulfate.

This is quite different from the pattern of manifestation of heretofore known activities of polysaccharide sulfates against herpes virus.

In view of the fact that the polysaccharides with higher molecular weight and their sulfate are known to have the high toxicities to human being and animals, the experimental evidence obtained in the present invention that the dextran sulfate with lower molecular weight show sufficient anti-AIDS activity, is extremely important in developing it as a medicament for prevention and therapy of the viral disease.

Among the above test substances, those which showed particularly strong anti-AIDS virus activities are dextran sulfate,  $\lambda$ -carrageenan, alginic acid sulfate, chitosan sulfate, chondroitin polysulfates, further sulfated chondroitin-4-sulfate and -6-sulfate, heparin, etc. having S-content more than 10%.

#### Example 10 (Anti-Friend leukemia virus (F-MuLV) activity

##### (Procedure 1)

Anti-FMuLV activity of dextran sulfate (molecular weight: 7000 - 8000, S-content: 17 - 20 %) was determined by an XC-plaque assay method. BALB3T3 cells were cultured in adhesive form in a 35 mm-dish at  $5 \times 10^4$  cells/dish (2 ml). After removing the culture medium, a fresh medium with or without indicated concentrations of the test substance (1 ml each) and 0.2 ml of the virus preparation were charged, and the cells were cultured overnight. On the following day, the culture media were replaced with those (2 ml) containing or not containing the above substances, the incubation was continued for three additional days to progress the infection and replication of the virus. After the removal of the medium, the further progression of viral replication was stopped by UV irradiation. To this dish, the suspension of XC-cells (2 ml) was added and cultured for three days and the plaque formation produced by the virus particle induced cell-fusion, was observed. The number of plaques was shown in the following Table.



TableAnti-Freind leukemia virus activity by Procedure 1

DS	Number of plaques	Inhibition
( $\mu\text{g/ml}$ )	per dish	(%)
Control	16	(0)
1	14	92
5	12	93
10	11	93
50	13	92
100	6	96
1000	0	100

As observed from the above Table, DS inhibited 90 % or more the formation of plaque at the concentrations of 1 - 100  $\mu\text{g/l}$ , indicating that the infection and replication of the virus was strongly inhibited. The plaque formation was not detected at 1,000  $\mu\text{g/ml}$  of DS.

DS at 1 - 100  $\mu\text{g/ml}$  did not show any cytotoxicity to BALB 3T3 cells.

(Procedure 2)

Procedure 1 was repeated except that after adsorption of the virus in the medium without DS, the non-adsorbed viruses were removed and the culture was carried out in the medium (2 ml) containing or not containing DS. The results are shown in the following table.

TableAnti-Friend leukemia virus activity by Procedure 2:

	DS	Number of plaques	Inhibition
	( $\mu\text{g/ml}$ )	per dish	(%)
	Control	35	(0)
	0.01	33	6
	0.1	19	45
	1	14	61
	10	17	52
	100	16	54
	500	0	100

The above results indicate that, also in Procedure 2, DS inhibited the infection and replication of the virus by about 60 % at the concentration of 1  $\mu\text{g}$ , and almost completely at 500  $\mu\text{g/ml}$ .

From the above results, it is evidenced that DS inhibits the infection and replication of the oncogenic virus (Oncovirinae) including F-MuLV, as well as the cytolytic virus (Lentivirinae) including AIDS-virus.

**Claims**

1. The use of a natural or synthetic oligo-or polysaccharide having at least one S-oxoacid group attached to the saccharic carbon atom through a linking group of lower molecular weight or a pharmaceutically acceptable salt thereof for the manufacture of a medicament for treatment of disease caused by retroviruses.
2. The use according to claim 1, wherein the said S-oxoacid group is sulfo group ( $-\text{SO}_3\text{H}$ ).
3. The use according to claim 1, wherein the said linking group is oxy group ( $-\text{O}-$ ) or imino group ( $-\text{NH}-$ ).
4. The use according to claim 1, wherein the said treatment is prevention or therapy of PGL, ARC, AIDS, ATL, or Kawasaki disease.
5. The use according the claim 1, wherein the said retroviruses are human retroviruses.
6. The use according to claim 5, wherein the said human retroviruses are selected from HIVs such as HTLV-III, LAV, and ARV.
7. The use according to claim 5, wherein the said human retroviruses are selected form HTLV-I, HTLV-II and Kawasaki disease causative retroviruses.
8. The use according to claim 1, wherein the said retroviruses are animal retroviruses.
9. The use according to claim 1, wherein the said retroviruses are selected from avian myeloblastosis virus and Friend murine leukemia virus.
10. The use according to claim 1, wherein the said treatment is prevention from the infection with retroviruses.
11. The use according to claim 10, wherein the said retroviruses are HIVs.
12. The use according to claim 1, wherein the said oligo-or polysaccharide has ability of inhibiting reverse transcriptase of the retroviruses.

13. The use according to claim 1, wherein the said oligo-or polysaccharide is natural or synthetic polysaccharide sulfate ester or a pharmaceutically acceptable salt thereof.

14. The use according to claim 13, wherein the said natural or synthetic oligo-or polysaccharide is a natural polysaccharide having at least one hydrogen sulfate group(-O-SO<sub>3</sub>H) obtained from a plant or a microorganism, or a synthetic polysaccharide having at least one hydrogen sulfate group(-O-SO<sub>3</sub>H) formed by esterifying a polysaccharide obtained from a plant or a microorganism with sulfating agent, or a pharmaceutically acceptable salt thereof.

15. The use according to claim 14, wherein the said synthetic polysaccharide is selected from dextran sulfate, alginic acid sulfate, lentinan sulfate and pururan sulfate.

16. The use according to claim 15, wherein the said dextran sulfate has a molecular weight between 500 and 2,000,000, ordinarily between 2,000 and 300,000, preferably between 2,000 and 10,000 and most suitably between 3,000 and 8,000.

17. The use according to claim 15, wherein the said dextran sulfate has a sulfur content between 5% and 22%, preferably between 10% and 20% and most suitably between 15% and 20%.

18. The use according to claim 14, wherein the said natural polysaccharide is selected from carrageenan, fucoidin.

19. The use according to claim 13, wherein the said natural or synthetic oligo-or polysaccharide is a natural polysaccharide having at least one sulfo group(-SO<sub>3</sub>H) obtained from an animal, or a synthetic polysaccharide having at least one sulfo group(-SO<sub>3</sub>H) formed by esterifying a polysaccharide obtained from an animal with sulfating agent, or a pharmaceutically acceptable salt thereof.

20. The use according to claim 19, wherein the said polysaccharide is a natural polysaccharide selected from mucopolysaccharide or a synthetic polysaccharide selected from mucopolysaccharide sulfate produced by esterifying a natural mucopolysaccharide with a sulfating agent.

21. The use according to claim 19, wherein the said polysaccharide is heparin or a pharmaceutically acceptable salt thereof.

22. The use according to claim 19, wherein the said polysaccharide is a natural polysaccharide selected from chondroitin sulfate, dermatan sulfate, heparitin sulfate, keratan sulfate, hyaluronic acid, teichronic acid, chitin and chitosan or synthetic polysaccharide selected from chondroitin polysulfate, dermatan polysulfate, heparitin polysulfate, keratan polysulfate, hyaluronic acid sulfate, teichronic acid sulphate, chitin sulfate and chitosan sulfate, or a pharmaceutically acceptable salt thereof.

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FIG.1

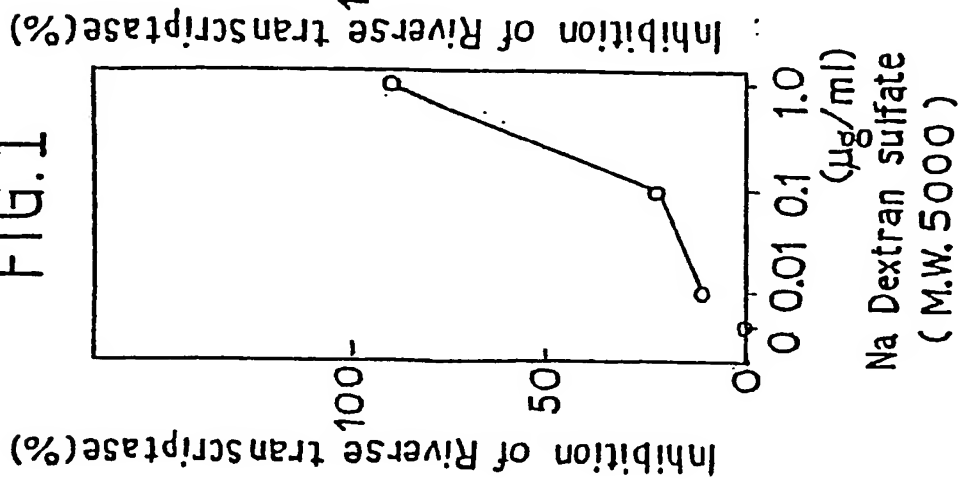


FIG.2

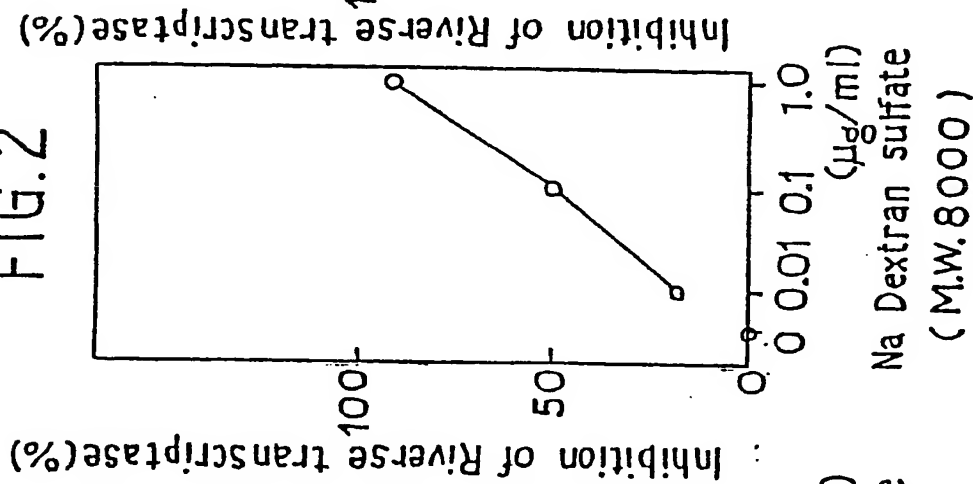


FIG.3

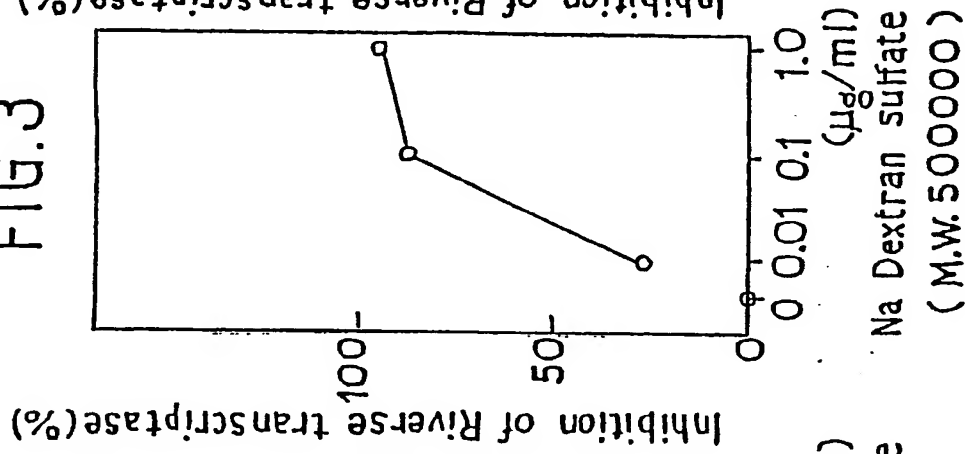
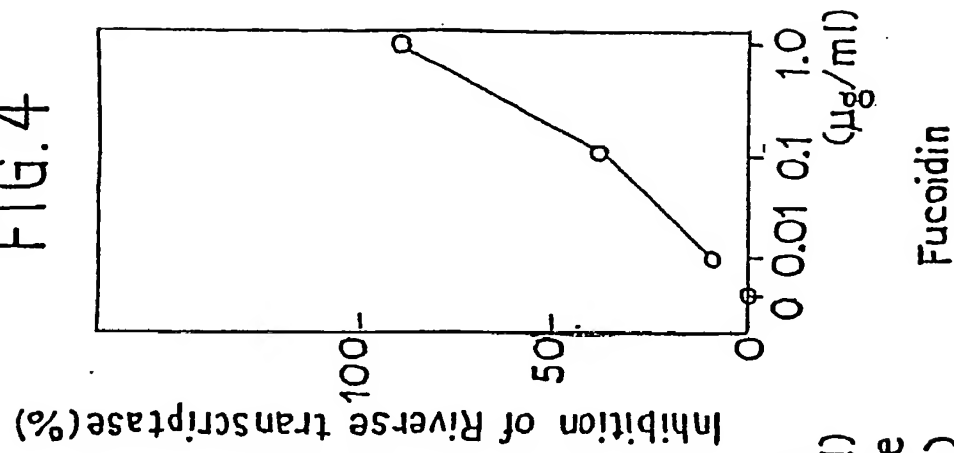


FIG.4



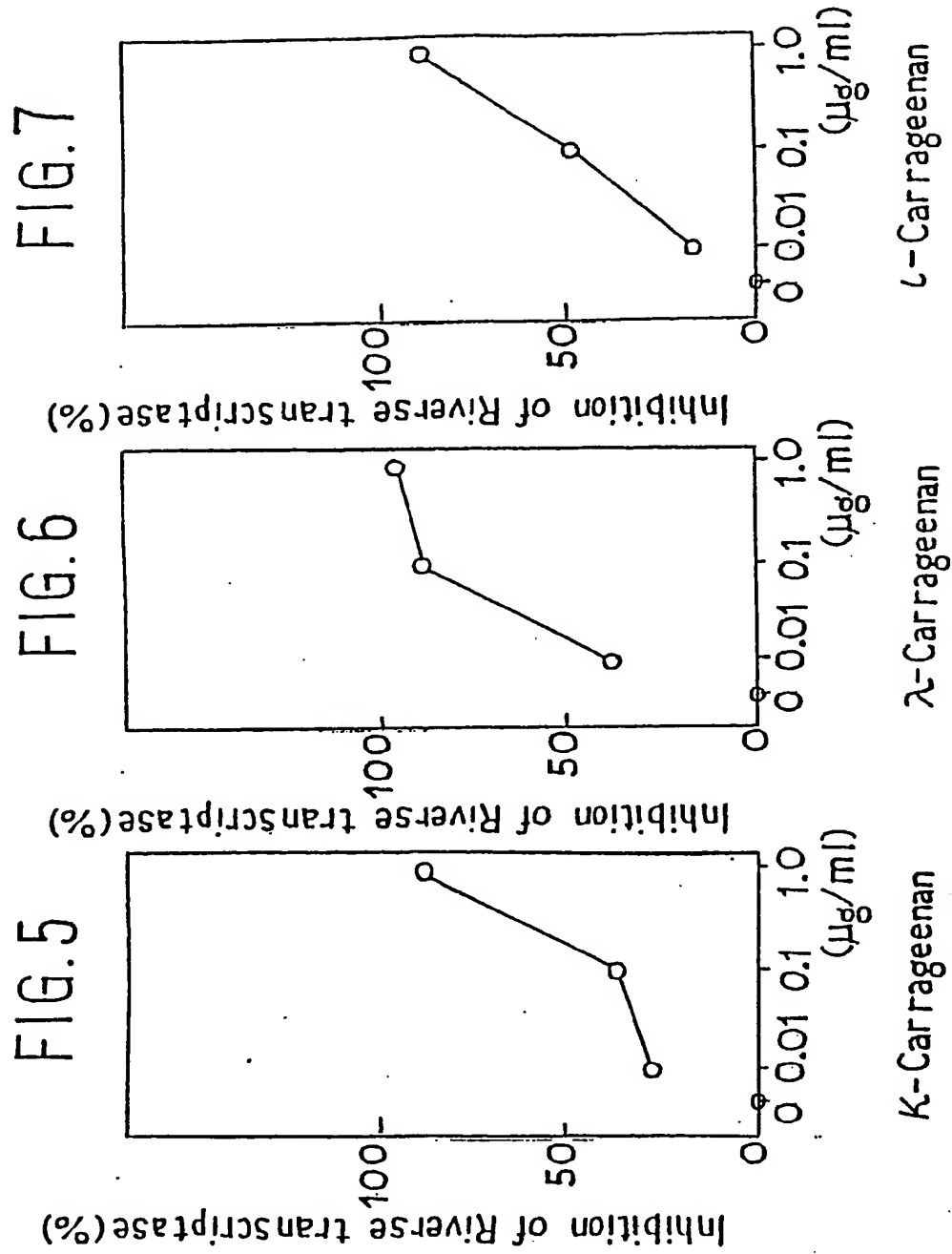


FIG. 8

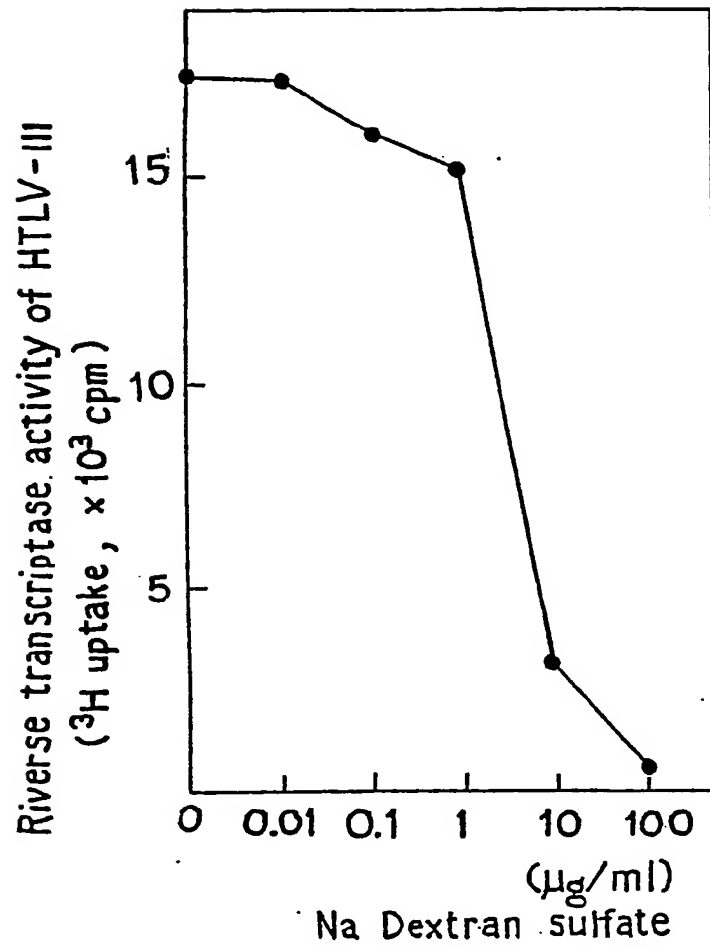


FIG.9

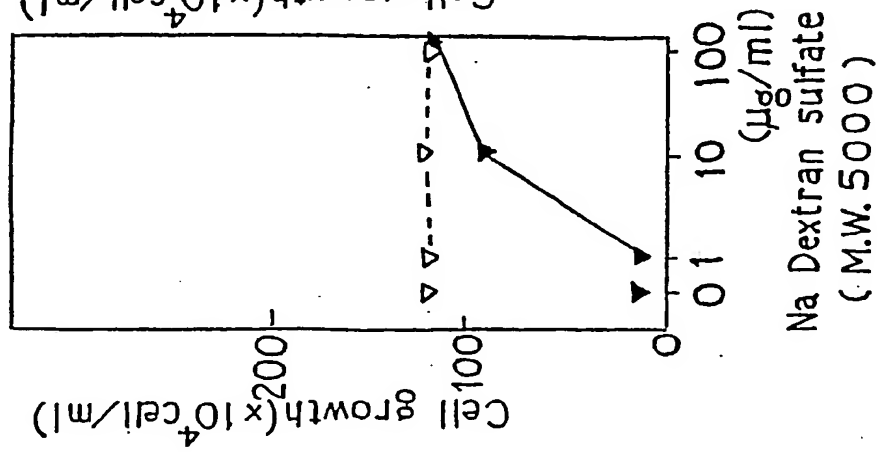


FIG.10

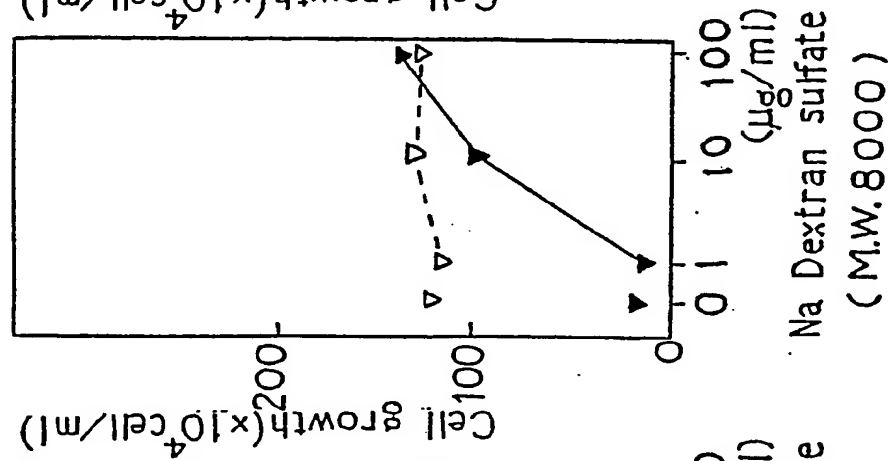


FIG.11

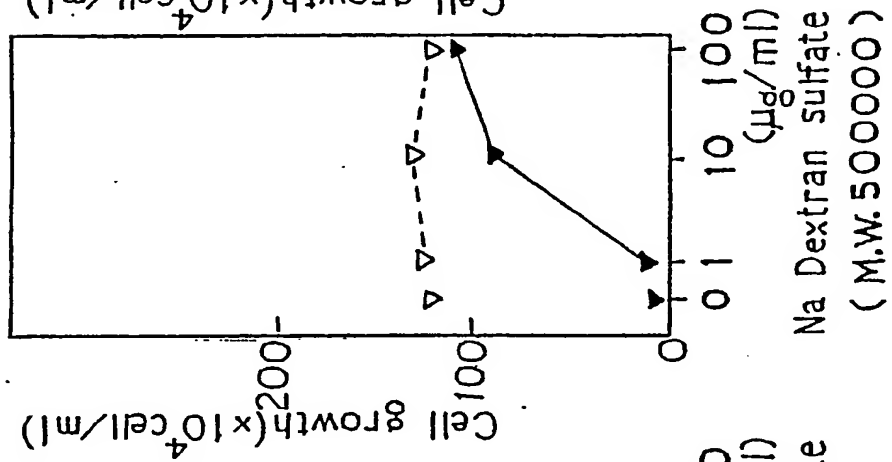


FIG.12

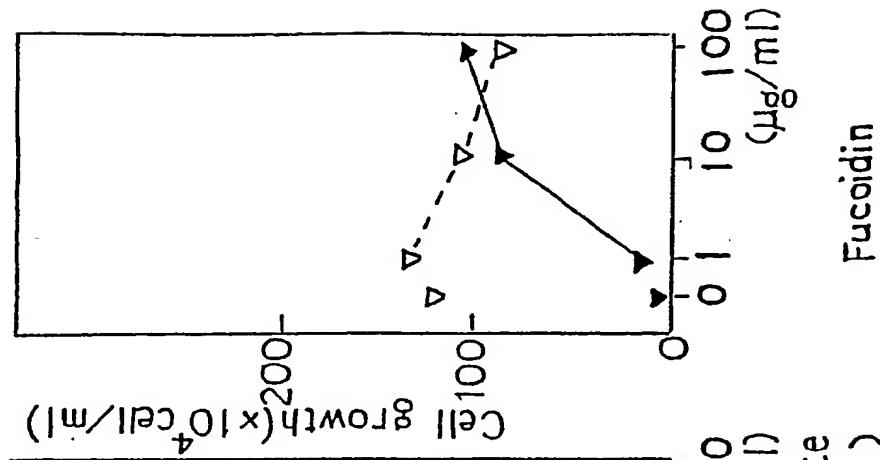


FIG. 13

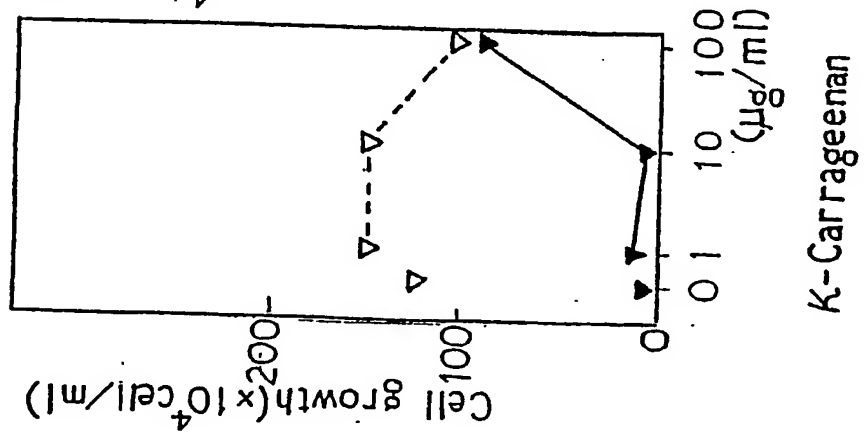


FIG. 14

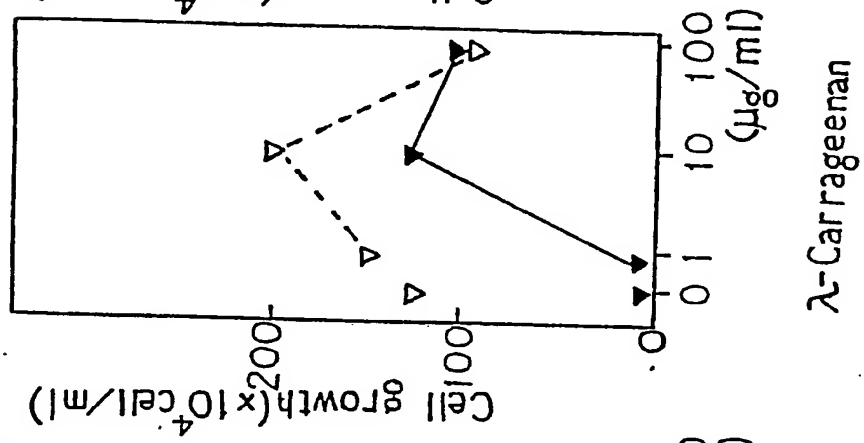


FIG. 15

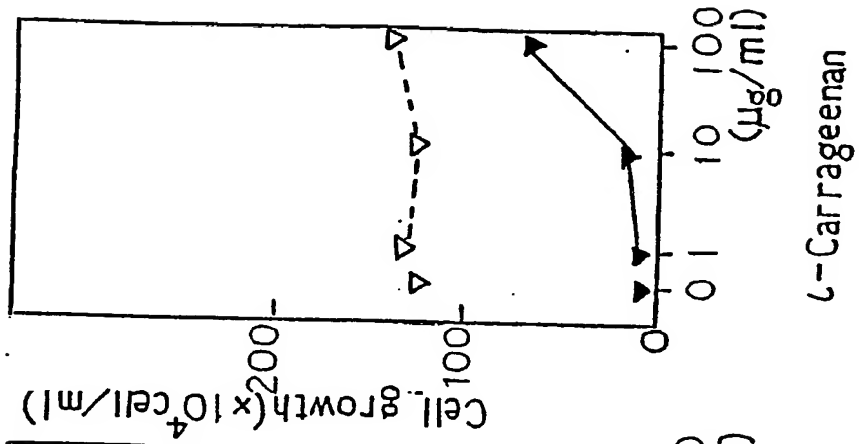




FIG.16

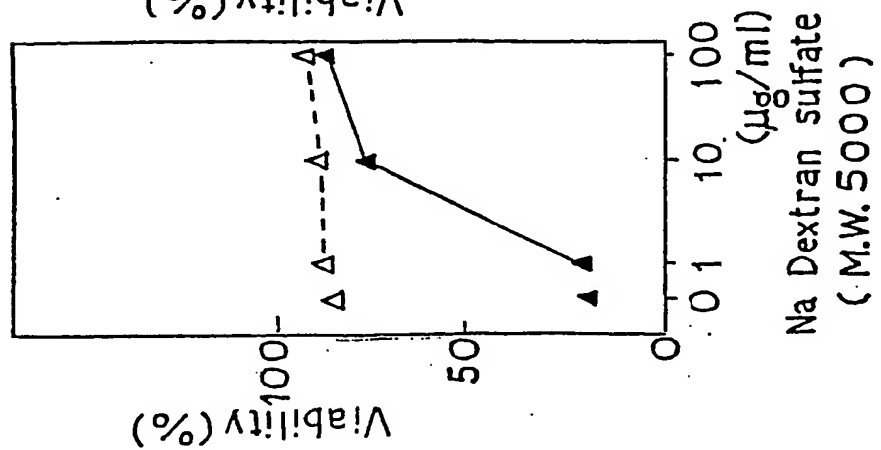


FIG.17

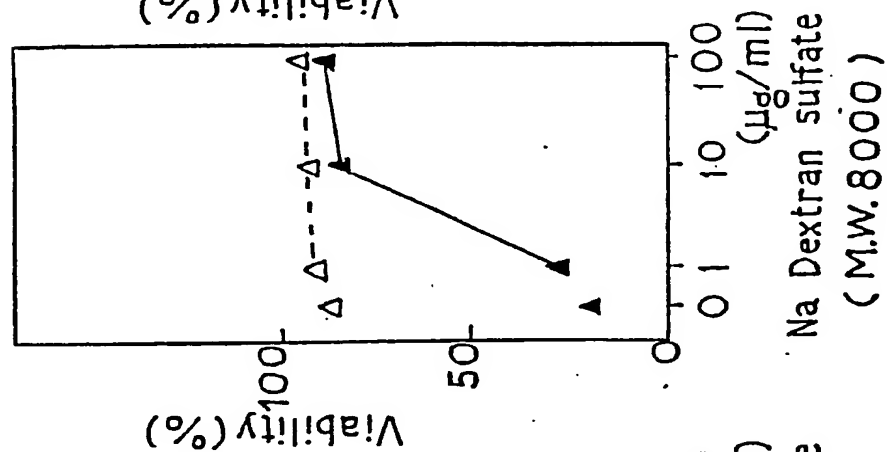


FIG.18

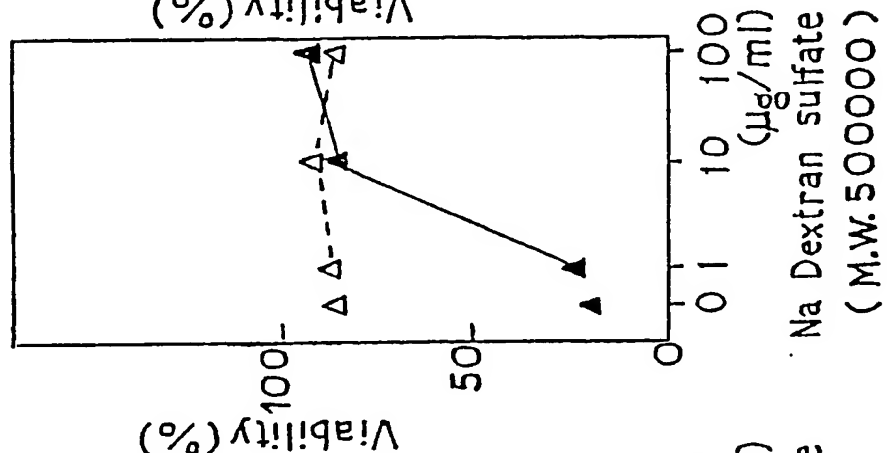


FIG.19

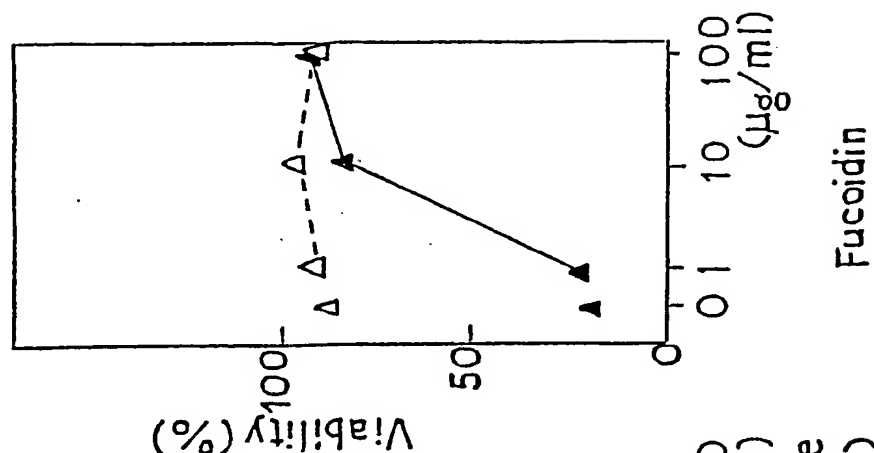


FIG. 20

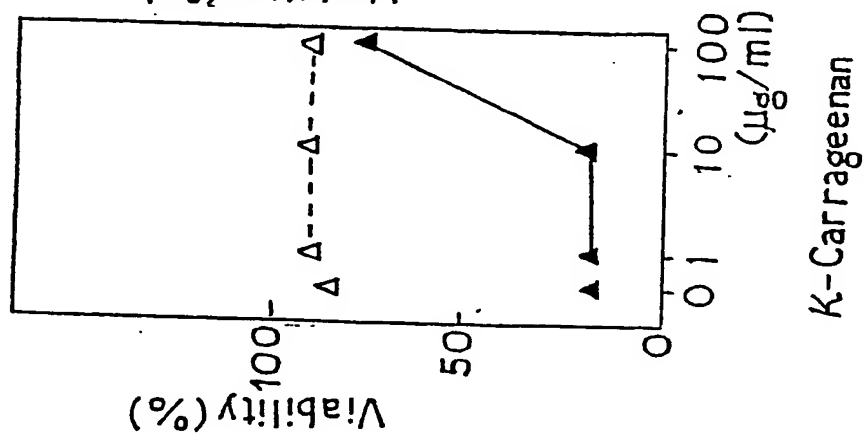


FIG. 21

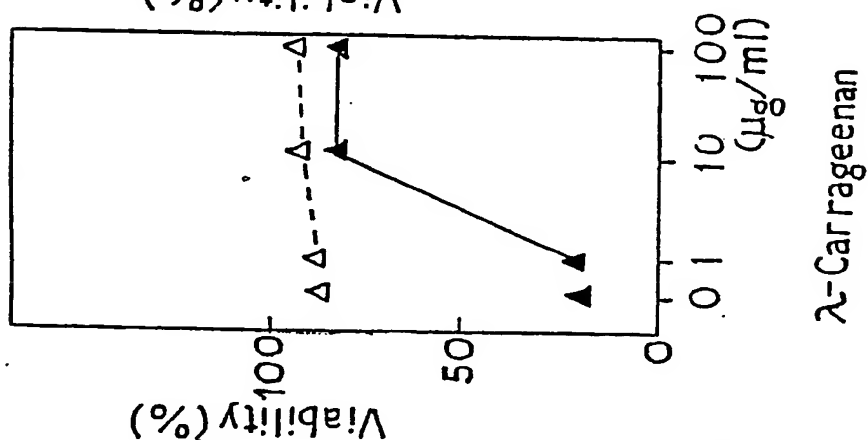


FIG. 22

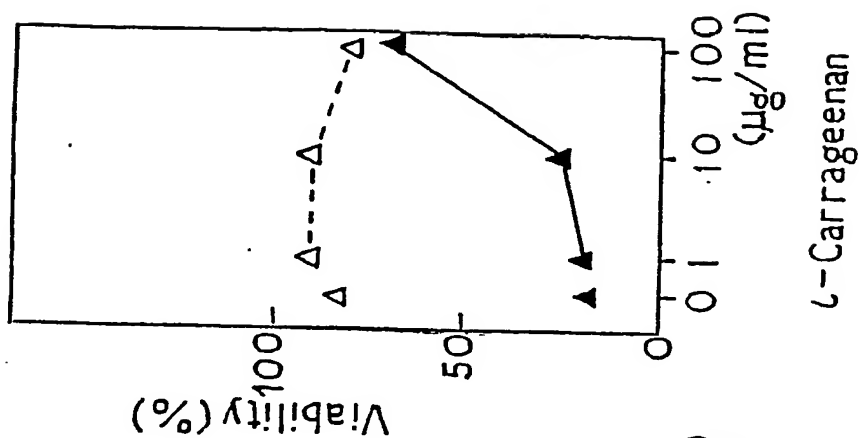


FIG. 23

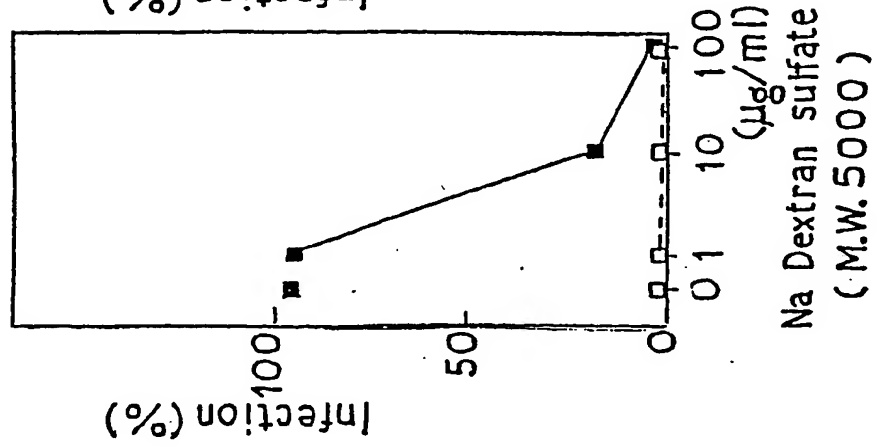


FIG. 24

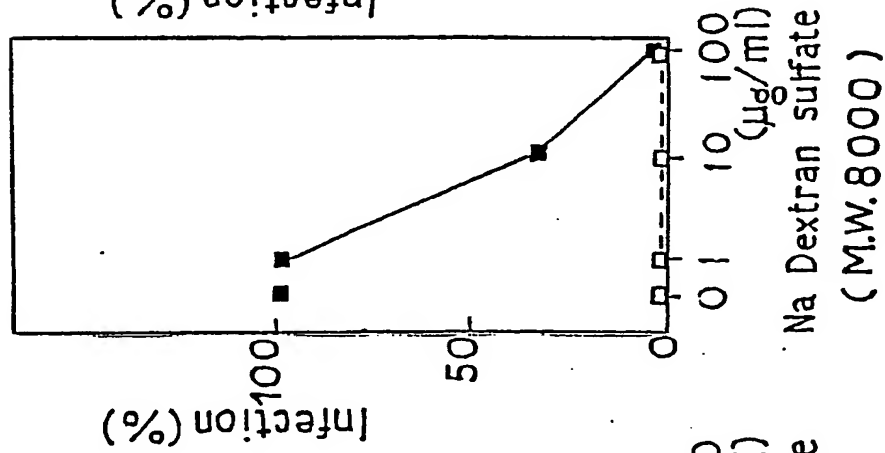


FIG. 25

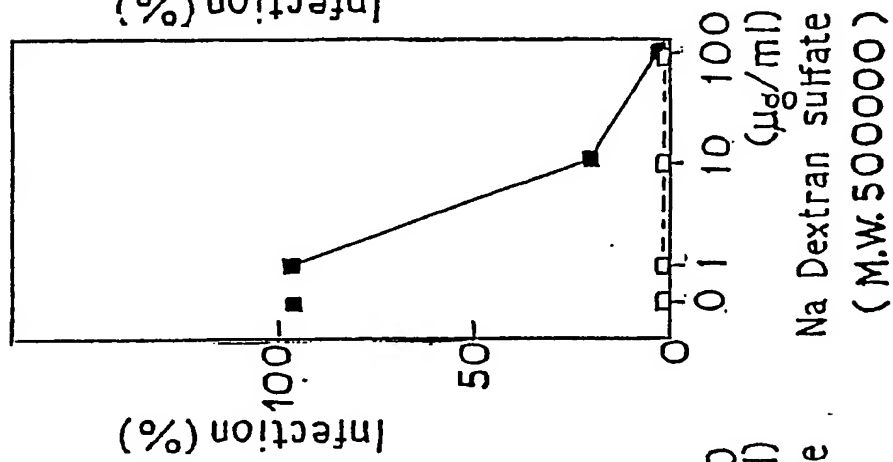
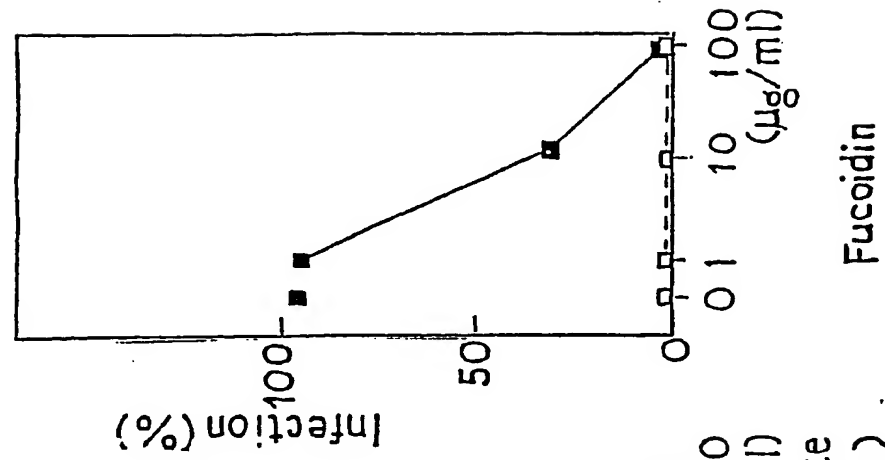


FIG. 26



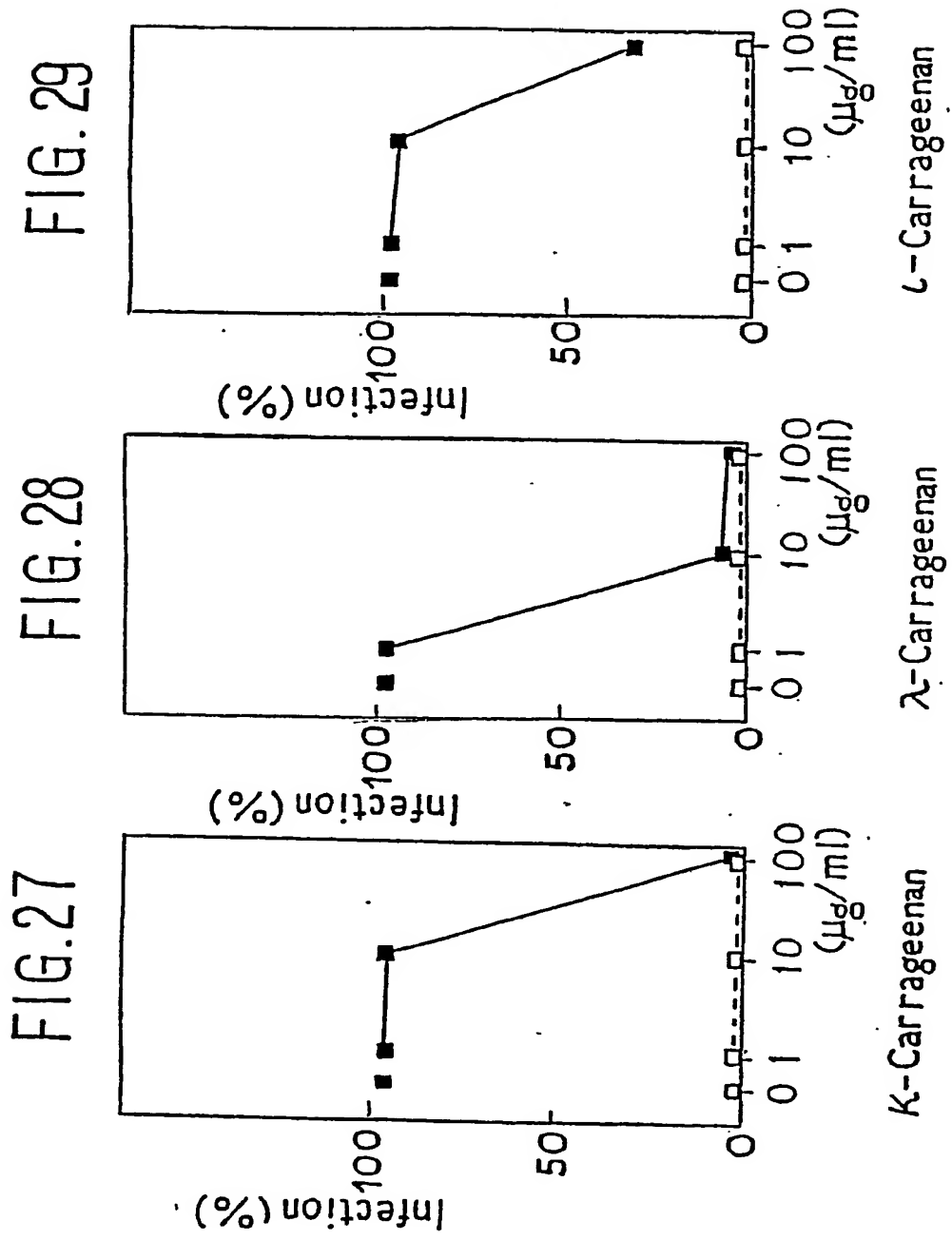
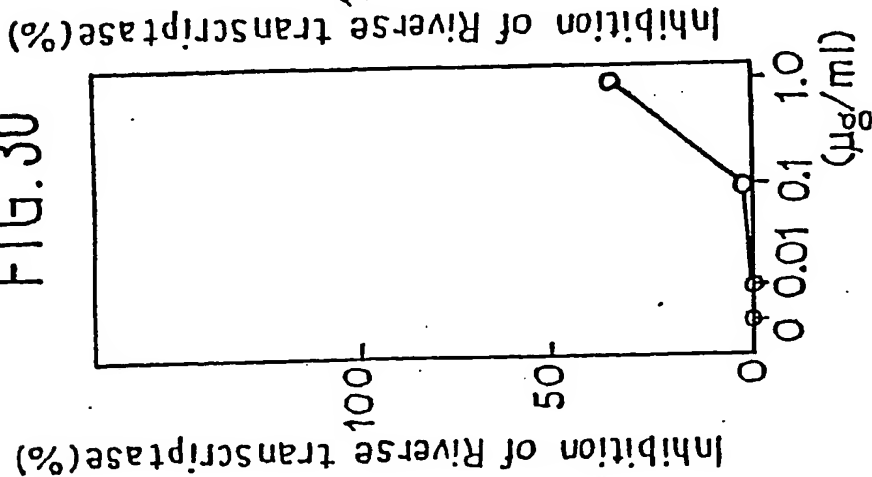
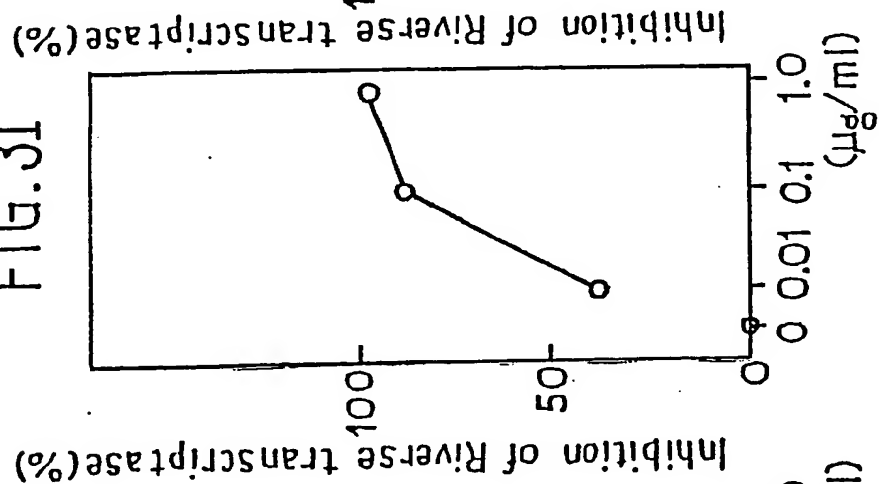


FIG. 30



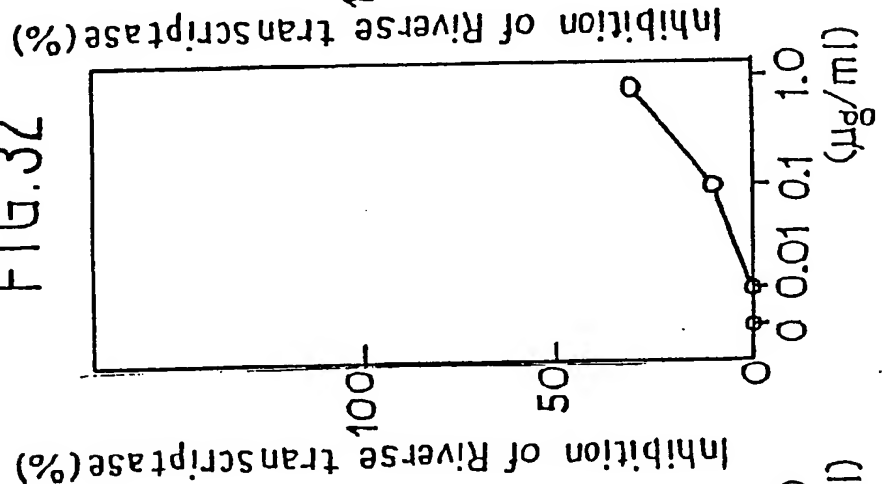
Na Chondroitin sulfate

FIG. 31



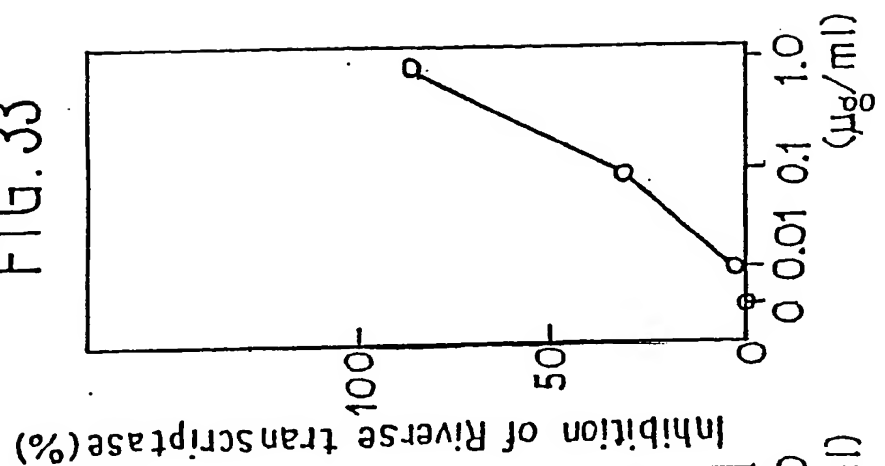
Na Chondroitin polysulfate

FIG. 32



Na Keratan sulfate

FIG. 33



Na Keratan polysulfate

FIG. 34

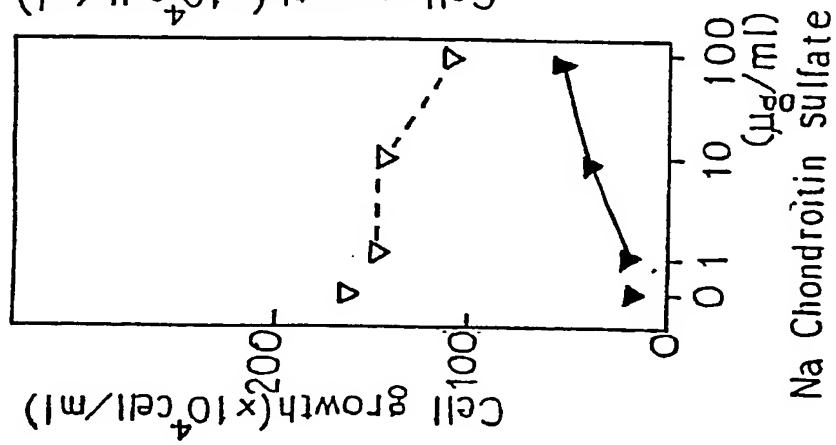


FIG. 35

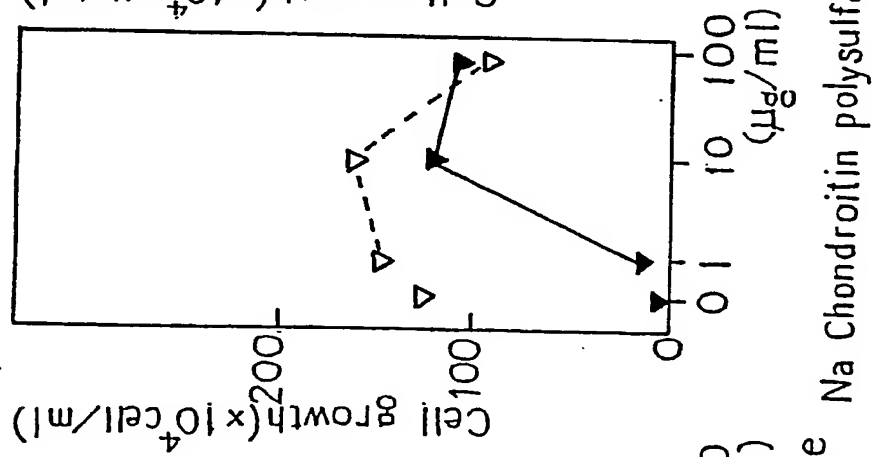


FIG. 36

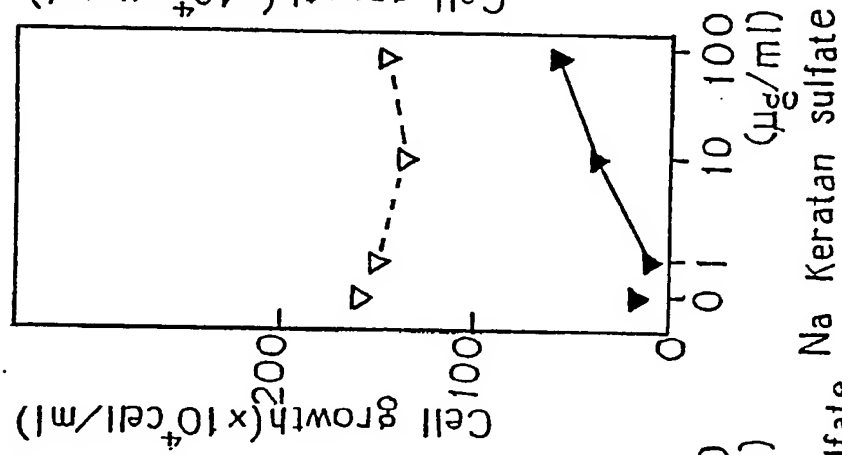


FIG. 37

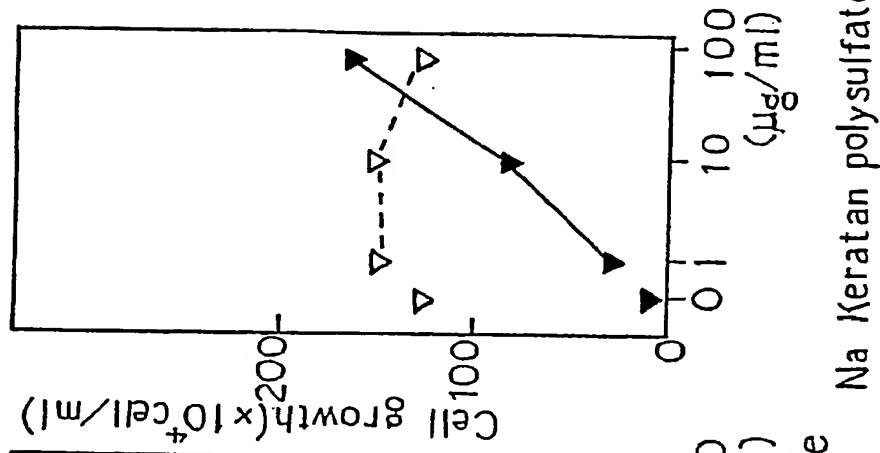


FIG. 38

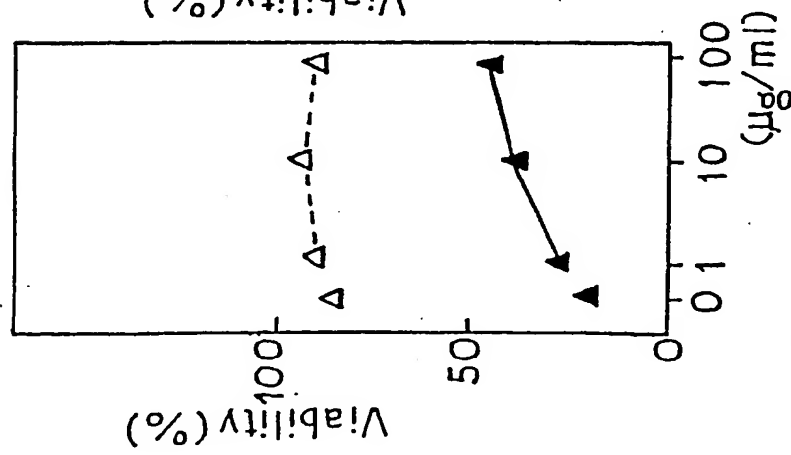


FIG. 39

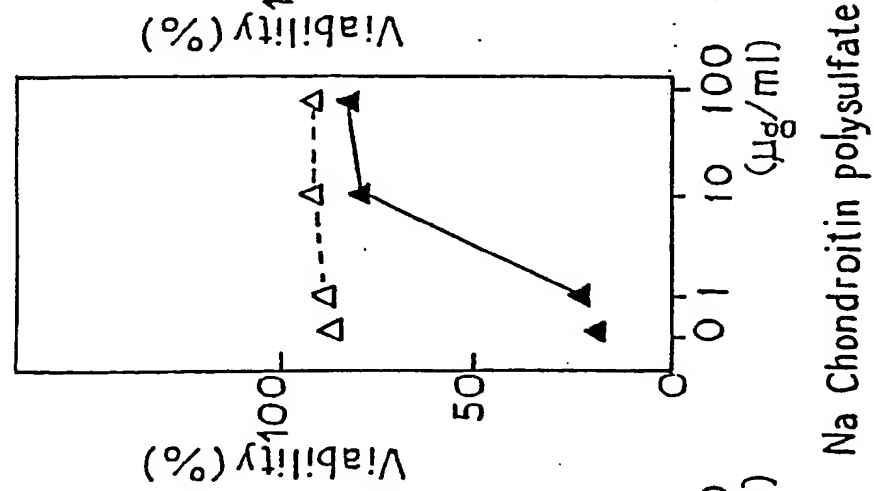


FIG. 40

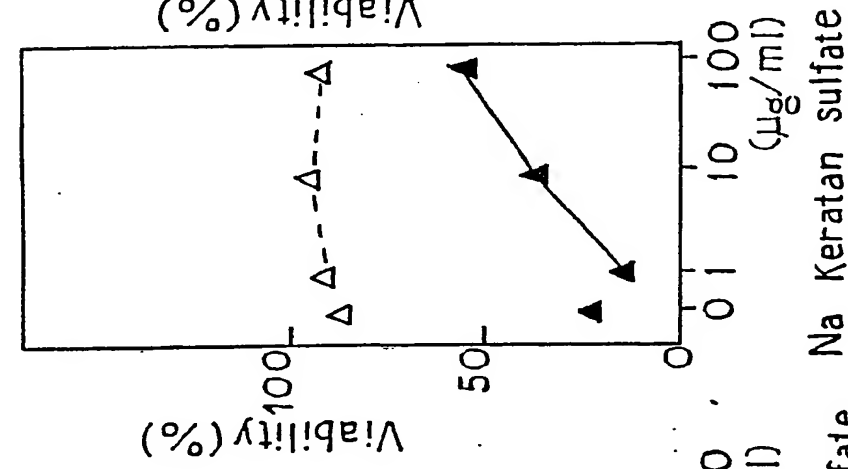


FIG. 41

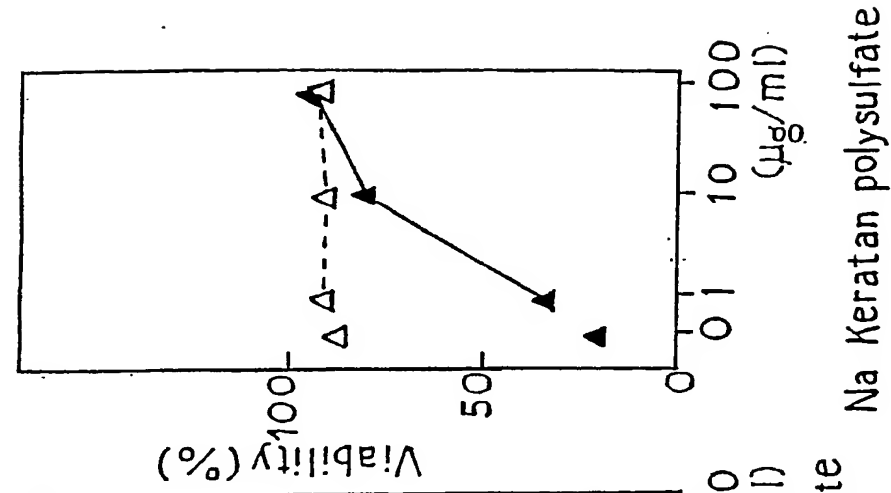
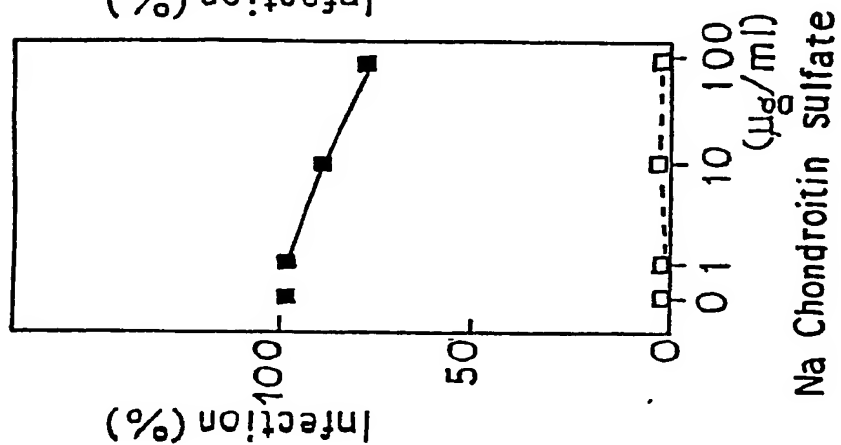
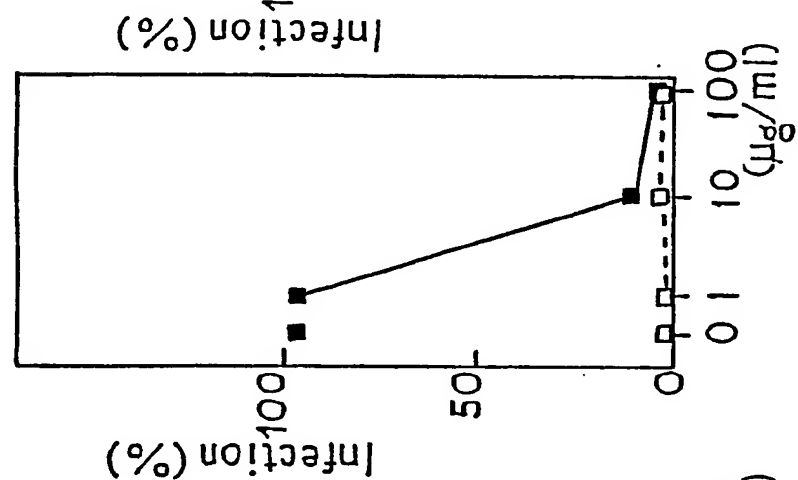


FIG. 42



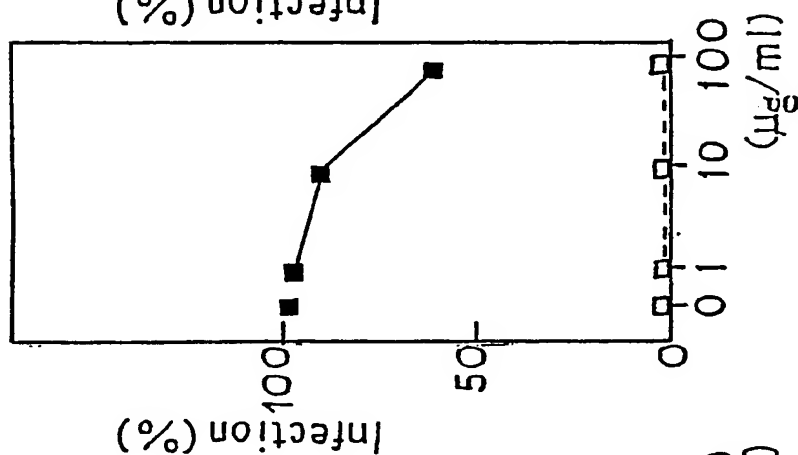
Na Chondroitin sulfate

FIG. 43



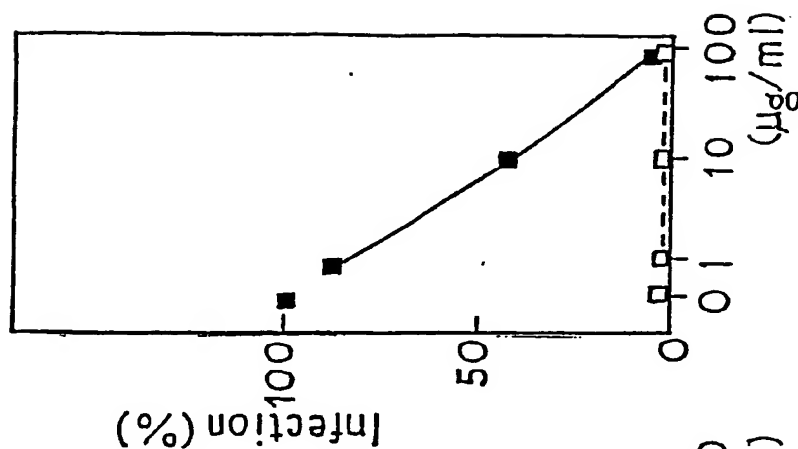
Na Chondroitin polysulfate

FIG. 44



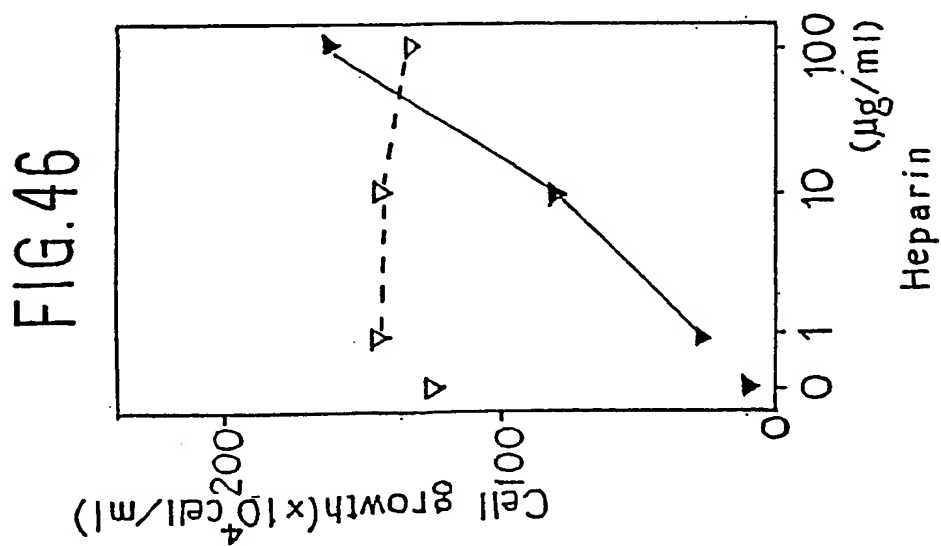
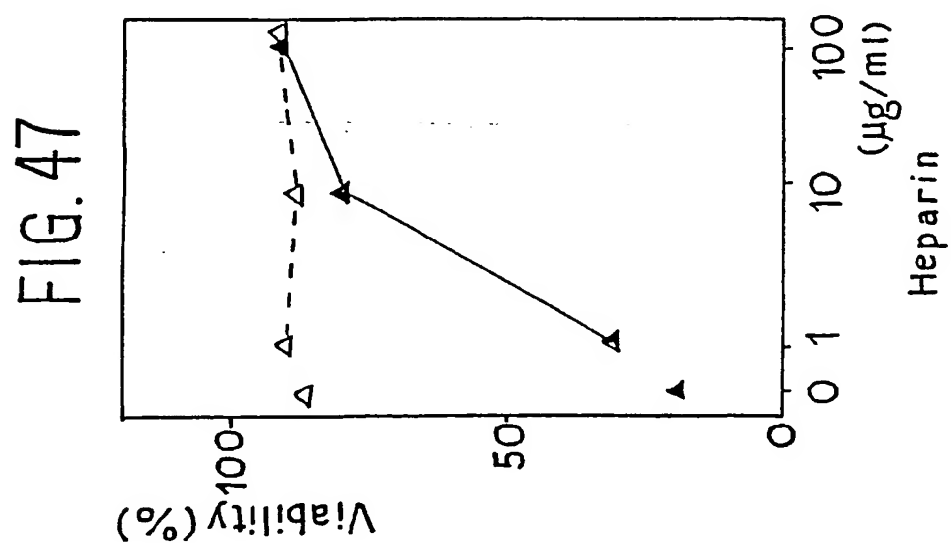
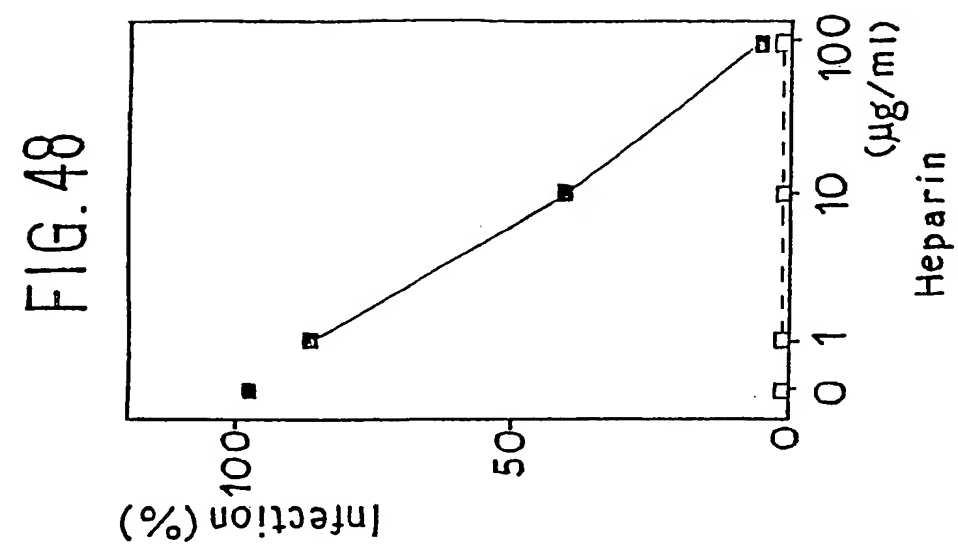
Na Keratan sulfate

FIG. 45



Na Keratan polysulfate





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Publication number:

**0 240 098  
A3**

12

## EUROPEAN PATENT APPLICATION

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54 Treatment of diseases caused by retroviruses.

57 The use of a natural or synthetic oligo- or polysaccharide having at least one S-oxoacid group attached to the saccharic carbon atom through a linking group of lower molecular weight or a pharmaceutically acceptable salt thereof for the manufacture of a medicament for treatment of diseases caused by retroviruses.

EP 0 240 098 A3



European Patent  
Office

# EUROPEAN SEARCH REPORT

Application Number

EP 87 30 0282

## DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.4)
E	EP-A-0 232 744 (MAX-PLANCK-GESELLSCHAFT ZUR FÖRDERUNG DER WISSENSCHAFTEN e.V.) * Whole document *	1-22	A 61 K 31/715 A 61 K 31/725
X,D	CANCER RESEARCH, vol. 38, no. 8, August 1978, pages 2401-2407; R.A. DICLOCCIO et al.: "Inhibition of deoxynucleotide-polymerizing enzyme activities of human cells and of simian sarcoma virus by heparin" * Whole article *	1-3,8, 10,12- 14,19- 21	
Y	Idem	4-7,9, 11,15- 18,22	
X	JOURNAL OF THE NATIONAL CANCER INSTITUTE, vol. 67, no. 4, October 1981, pages 899-910; J.L. ROJKO et al.: "Determinants of susceptibility and resistance to feline leukemia virus infection. II. Susceptibility of feline lymphocytes to productive feline leukemia virus infection" * Whole article *	1	
Y	Idem	2-22	
Y	J. GEN. VIROL., vol. 65, 1984, pages 1325-1330, SGM, GB; B. EHLERS et al.: "Dextran sulphate 500 delays and prevents mouse scrapie by impairment of agent replication in spleen" * Whole article *	4-7,9, 11,15- 18,22	
The present search report has been drawn up for all claims			
Place of search THE HAGUE		Date of completion of the search 08-02-1989	Examiner FOERSTER W.K.
CATEGORY OF CITED DOCUMENTS			
X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document			
T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons & : member of the same patent family, corresponding document			



DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl. 4)
Y	JOURNAL OF THE NATIONAL CANCER INSTITUTE, vol. 51, no. 5, November 1973, pages 1705-1707; B.L. KLEHL et al.: "Anticoagulants as inhibitors of reverse transcriptase activity" * Whole article *	1-22	
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Place of search THE HAGUE		Date of completion of the search 08-02-1989	Examiner FOERSTER W.K.
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